REMARKS/ARGUMENTS

Claims 36-49 are currently pending. Claims 36, 38, and 44-46 are amended to correct a minor typographical error by substituting "GlcNAc" for "GLcNAc." Because these amendments correct an obvious error, no new matter is added.

All pending claims stand rejected as allegedly not enabled by the specification under 35 U.S.C. § 112, first paragraph. Reconsideration and withdrawal of this rejection are respectfully requested in view of the remarks set forth below.

Interview Summary

Applicants thank the Examiner for the teleconference of May 3, 2006, with Kevin Bastian and the undersigned, during which enablement issues pertaining to *in vivo* use of glycosyltransferase inhibitors and the submission of proofs relating thereto were discussed. Applicants' representatives understand that the Examiner would consider evidence of *in vivo* inhibition of other glycosyltransferases as evidencing predictability of achieving *in vivo* Core 2 GlcNAc transferase inhibition in accordance with the present invention. This response serves to enter these proofs, together with additional argument, further demonstrating enablement of the pending claims. While no specific agreement was reached, the Examiner indicated that the remaining enablement rejection would be reconsidered in light of Applicants' evidentiary submission.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 36-49 remain rejected under 35 U.S.C. § 112, first paragraph, as allegedly not enabled by the specification. The Examiner, having already accepted the specification's demonstration that *in vivo* inhibition of Core 2 GlcNAc transferase would lead to inhibition of inflammation,[1] also now accepts that the passive diffusion of small molecules into cells was

[1] *See* Office Action dated August 24, 2005, at p. 10.

predictable as of the specification's filing date, as shown by Camenisch *et al.*[2] In maintaining the enablement rejection, however, the Examiner asserts that "the correlation between the ability to predict the passive diffusion of a molecule under ... controlled *in vitro* conditions ... and the ability to mimic these conditions *in vivo* is lacking."[3] In particular, the Examiner alleges that unpredictability lies in whether the inhibitors can be adequately delivered to the desired mammalian target cells *in vivo*, as well as to "the desired subcellular organelles."[4] Based on this alleged unpredictability in targeting, the Examiner contends that it is unpredictable "whether effective inhibitor concentrations can be obtained *in vivo* to inhibit the target enzyme."[5]

Initially, and as indicated during the Examiner Interview, Applicants note that the desired mammalian target cells in the instant case are leukocytes, which are present in the bloodstream. As described in the specification as filed, the target enzyme, Core 2 GlcNAc transferase, mediates the synthesis of selectin ligands,[6] which are involved in the interaction between leukocytes and the vascular endothelium and, therefore, play a role in inflammatory responses.[7] Accordingly, because the target cells are present in the bloodstream, effective inhibitor concentrations can be readily achieved, for example, by known methods for systemic administration of pharmaceutical agents, including, *e.g.*, i.v. administration or absorption through the gut. Once present in the bloodstream, inhibitors having the ability to passively cross cellular membranes (as predicted, for example, by the method of Camenisch *et al.*) would be expected to passively diffuse into target leukocytes and into the appropriate subcellular organelle to exert a physiological effect.

As evidence that inhibitors of glycosyltransferases are, as a class of inhibitors, capable of targeting the appropriate subcellular organelle of cells in the bloodstream to exert a corresponding physiological effect *in vivo*, Applicants respectfully refer the Examiner to Exhibits D and E, each attached hereto. As indicated above, it is Applicants' understanding that the Examiner would consider such evidence as supporting predictability for achieving *in vivo*

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[2] *Eur. J. Pharm. Sci.* 6:317-24, 1998. *See* Office Action dated 2/22/06 at p. 3.
[3] *Id.*
[4] *Id.* at p. 4.
[5] *See id.*
[6] *See* Application at p. 40, ll. 9-19.

inhibition of Core 2 GlcNAc transferase in accordance with the present invention. Each of these evidentiary references are discussed in detail below, with particular reference to the Examiners remarks raised in the last Office Action and during the Examiner Interview.

First, with respect to the Examiner's concern regarding targeting of the inhibitor to the appropriate subcellular organelle, a demonstration of a substrate analog's ability to specifically inhibit glycosylation in intact cells, whether *in vitro* or *in vivo*, demonstrates the ability of the inhibitor to enter the cell and the subcellular organelle where the target enzyme is present. Also, it was noted during the Examiner Interview that glycosyltransferases, as a class of enzymes and including Core 2 GlcNAc transferase, reside and exert their effects primarily in the Golgi apparatus.[8] Therefore, Applicants further submit that the ability of a glycosyltransferase substrate analog to exert specific inhibitory effects on intact cells evidences the predictability of targeting Core 2 GlcNAc transferase inhibitors to the Golgi region to achieve corresponding biological effects.

Accordingly, as an exemplary demonstration of the ability of glycosyltransferase substrate analogs to enter the Golgi region and exert specific inhibitory effects on mammalian cells, Applicants have attached Exhibit D[9] (hereinafter "Morin *et al.*"), which describes the effects of tunicamycin, an inhibitor of dolichol-linked oligosaccharide synthesis, in leukemic L1210 cells. As described in the specification, and as the Examiner is aware, tunicamycin is an inhibitor of N-acetylglucosaminyltransferase and is an analog of UDP-GlcNAc, the donor substrate for this glycosyltransferase.[10] As described in Morin *et al.*, tunicamycin was found to specifically inhibit the incorporation of a number of sugars into glycoproteins in L1210 leukemia cells.[11]

As to the Examiner's concerns regarding targeting of the inhibitor to mammalian cells *in vivo*, Applicants believe the predictability of *in vivo* targeting is also supported by Morin

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[7] *See id.* at p. 2, ll. 1-20.
[8] As described in the specification, "preferred glycosyltransferase inhibitors of the present invention have the ability to cross the cell membrane and enter the Golgi apparatus" (*see* specification at p. 23, ll. 3 & 4).
[9] Morin *et al.*, *J. Cell. Physiol.* 114:162-172, 1983.
[10] *See* specification at p. 21, ll. 27-29.
[11] *Id.* at Abstract; *see also* p. 164, 2nd col. 1st full paragraph.

et al. As discussed above, targeting to cells in the bloodstream is already believed predictable in view of Camenish *et al.*, which demonstrates the ability to determine passive diffusion of small molecules based on readily available biophysical parameters. Morin *et al.* provides particular confirmation that glycosyltransferase substrate analogs can enter into intact cells to exert specific inhibitory effects. Moreover, because Morin's L1210 leukemic cells are derived from mammalian leukocytes, these cells are representative of the type of cells targeted in accordance with the present invention. Therefore, Applicants again submit that, once present in the bloodstream, glycosyltransferase substrate analogs having the ability to passively cross cellular membranes would be expected to enter into target leukocytes (also in the bloodstream) and into the Golgi region to exert a physiological effect.

As a further demonstration that *in vivo* delivery of a glycosyltransferase inhibitor can be achieved, along with corresponding treatment effects associated with cellular interactions, Applicants refer the Examiner to Exhibit E[12] (hereinafter "Kijima-Suda *et al.*"), which describes the inhibition of blood-borne tumor cell metastasis *in vivo* upon i.v. administration of a sialyltransferase substrate analog inhibitor, KI-8110. As discussed in Kijima-Suda *et al.*, KI-8110 is a sialic acid:nucleoside conjugate having sialyltransferase inhibiting activity that specifically depends on the acceptor.[13] KI-8110 was used in Kijima-Suda's studies as a means for inhibiting sialylation of the tumor cell surface, which had been correlated to metastatic potential.[14] Inhibition of tumor cell metastasis in mice, as well as prolongation of survival, were observed when NL-17 or NL-44 tumor cells where intravenously injected into mice, followed by injection of KI-8110.[15] This effect was observed even without *in vitro* pretreatment of the NL-17 or NL-44 cells with KI-8110 (*i.e.*, where the tumor cells were exposed to the inhibitor only *in vivo*).[16] Applicants submit that Kijima-Suda's studies, which involve the interaction of blood-borne tumor cells with, *e.g.*, vascular, endothelial, and circulating host cells,[17] are particularly relevant to enablement of the presently claimed invention, as the instant application demonstrates

[12] Kijima-Suda *et al.*, *Cancer Research* 46:858-862, 1986.
[13] *Id.* at p. 860, 2nd col., 1st full paragraph.
[14] *See id.* at p. 858 (Abstract and Introduction).
[15] *See id.* at Abstract; p. 859, 1st col., last paragraph, and 2nd col., last paragraph.
[16] *See id.* at p. 859, 2nd col., last paragraph.
[17] *See id.* at p. 860, 2nd col., top.

that inhibition of Core 2 GlcNAc transferase perturbs selectin-mediated interactions of leukocytes with vascular endothelial cells, thereby inhibiting inflammation.[18]

For the reasons above, in addition to reasons previously of record, Applicants submit that achieving effective concentrations of C2 GlcNAc-T inhibitors *in vivo*, so as to achieve the claimed treatment effect, to be predictable in view of the specification's teachings and the state of the art. The Examiner has accepted specification's demonstration that *in vivo* inhibition of Core 2 GlcNAc transferase would lead to inhibition of inflammation. Further, the particular class of inhibitors claimed – analogs of a glycosylatransferase substrate – has been used successfully *in vivo* for other indications. In addition to their use as antivirals and antibacterials, such inhibitors have been shown to exert physiological effects on mammalian cells, including *in vivo*. Morin *et al.* and Kijima-Suda *et al.*, for example, demonstrate that this class of inhibitors can target mammalian cells, including cells present in the bloodstream, to inhibit oligosaccharide synthesis and protein glycosylation reactions. Kijima-Suda *et al.*, in particular, shows that such inhibitors can be used *in vivo* so as to achieve specific enzyme inhibition in cells present in the bloodstream to achieve a corresponding treatment effect. Accordingly, those skilled in the art would view the use of this class of molecules for inhibiting inflammation, by *in vivo* competitive inhibition of Core 2 GlcNAc transferase expressed on circulating leukocytes in the blood, to be predictable.

In light of the above, Applicants believe claims 36-49 to be enabled by the specification as filed under 35 U.S.C. § 112, first paragraph. Withdrawal of the rejection is respectfully requested.

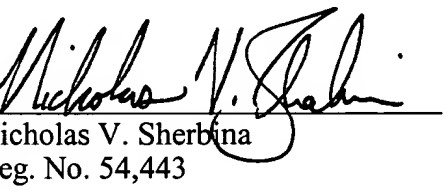
[18] *See* specification at, *e.g.*, p. 45, l. 26 to p. 47, l. 7.; and p. 49, ll. 3-14.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Respectfully submitted,

Date: June 5, 2006 By: 

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The Biochemical and Ultrastructural Effects of Tunicamycin and *D*-Glucosamine in L1210 Leukemic Cells

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Tunicamycin was found to specifically inhibit the incorporation of a number of sugars into L1210 leukemia cell glycoproteins. This inhibition of glycoprotein biosynthesis led to a cessation of cell growth which was reversible in a dose-dependent and time-dependent manner. After removal of the antibiotic from L1210 cell cultures resumption of sugar incorporation preceded that of thymidine incorporation and the recovery of cell growth. The treatment of cells with tunicamycin resulted in a significant increase in the intracellular pool of UDP-N-acetylglucosamine which occurred concurrently with alterations in cell ultrastructure including distentions of the endoplasmic reticulum and nuclear membranes. Similar ultrastructural changes and increases in the intracellular pools of UDP-sugars were observed in L1210 cells exposed to 5 mM D-glucosamine, which suggested that the antiproliferative effects of tunicamycin may be related to the accumulation in the endoplasmic reticulum of one or more nucleotide sugar precursors of asparagine-linked glycoprotein biosynthesis. However, the biological effects of tunicamycin could be distinguished from those caused by *D*-glucosamine. Exposure of L1210 cells to tunicamycin resulted in specific alterations in the biochemical composition of the plasma membrane and in the inhibition of cellular agglutination by wheat germ agglutinin which were not apparent following exposure to equitoxic concentrations of the aminosugar. These studies, together with those which demonstrated that recovery of the cellular capacity to synthesize glycoproteins was obligatory for the recovery of cellular proliferation in tunicamycin-treated cells, suggested that inhibition of the synthesis of glycoproteins was the major factor limiting L1210 leukemic cell proliferation.

Tunicamycin (TM) represents a family of closely related antibiotics containing N-acetylglucosamine, a novel 11-carbon sugar tunicamine, a uracil moiety, and a fatty acid residue which, by virtue of its microheterogeneity, gives rise to the physically distinct (Ito et al., 1980) but biochemically similar (Keenan et al., 1981) components within this class of agents. Tunicamycin specifically inhibits the initial biosynthetic step in the assembly of dolichol-linked oligosaccharides (Tkacz and Lampen, 1975; Heifetz et al., 1979) and, accordingly, this antibiotic has been employed in attempts to elucidate the role of asparagine-linked glycoprotein synthesis in secretory processes (Hickman et al., 1977; Struck et al., 1978), plasma membrane turnover (Olden et al., 1978; Damsky et al., 1979), tumor cell immunogenicity (Morin and Bernacki, 1979), and metastasis (Irimura et al., 1981). The potent activity of tunicamycin has been demonstrated in terms of both its antiviral activity (Ogura et al., 1977) and its cytotoxicity in bacterial and eukaryotic cell systems (Tkacz, 1980). There have been no studies, however, in which the direct inhibition of asparagine-linked glycoprotein biosynthesis and the secondary or resultant perturbations of cellular metabolism have been examined in terms of their relative contributions to the cytotoxicity of this agent.

In these studies, we have described the biochemical and ultrastructural effects of tunicamycin and their reversibility in L1210 leukemic cells. A comparison of its biological effects was made with those caused by *D*-glucosamine, a sugar capable of trapping uridylate (Decker and Keppler, 1974) and inhibiting glycocojugate biosynthesis (Kornfeld, 1964).

MATERIALS AND METHODS

Cell culture, growth inhibition, and cell survival studies

All experiments were performed with murine leukemia L1210 cells maintained in logarithmic growth as suspen-

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0021-9541/83/1142-0162$03.50

Exhibit D

sion cultures in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 16 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and 8 mM morpholinopropane sulfonic acid (MOPS) (pH 7.35) in a humidified atmosphere of 5% CO_2 at 37°C. Stock cultures were verified as mycoplasma-free by direct culture under anaerobic conditions and by the absence of both uridine phosphorylase activity (Levine, 1972) and extranuclear fluorescence with Hoechst stain 33258 (Chen, 1977). Experimental cultures were grown in the presence of pencillin (50 units/ml) and streptomycin (50 μg/ml). The growth inhibitory activity of tunicamycin was determined with cells grown, from starting inocula of 5×10^4 cell/ml in quadruplicate, in stoppered glass culture tubes. Forty-eight hours later cell growth in complete RPMI 1640 medium was assessed with a model Z_f Coulter particle counter. Effects of TM on cell growth were quantitated by subtraction of the initial cell inocula (5×10^4 cells/ml, for both untreated and treated cells) from the 48-hr cell counts and the final data was expressed as a percentage of control cell growth. An ID_{50} (dose of TM necessary to inhibit cell growth by 50%) was obtained from a plot of cell growth versus TM concentration. The survival or viability of TM-treated cells was evaluated on the basis of the ability of TM-treated cells to form colonies during a 4-day period of growth, in a semisolid agarose matrix (Kinahan et al., 1980). After a 48-hr exposure of cells to TM in RPMI 1640 medium as described above, a log dose-response curve was generated and the PE_{50} value, which represents the concentration of tunicamycin resulting in 50% inhibition of cell plating efficiency (or colony formation in soft agar) was determined.

Incorporation of macromolecular precursors

The effects of exposure of L1210 cells to tunicamycin or *D*-glucosamine on the concurrent incorporation of radiolabeled precursors of protein, DNA, RNA, or glycoconjugate biosynthesis were determined using 2-ml aliquots of cells (3.0×10^5 cells/ml) in warmed growth medium. Radiolabeled precursors were added to the appropriate tubes in the following amounts: 0.25 μCi of *L*-[3,4,5-^{3}H]-leucine (final specific activity, 0.66 mCi/mole), 0.25 μCi of [methyl-^{3}H]-thymidine (72 Ci/mole), 1.25 μCi of *D*-[2-^{3}H]-mannose (0.63 Ci/mole), 2 μCi of *L*-[5,6-^{3}H]-fucose (1 Ci/mole), 3.0 μCi of *D*-[^{14}C-(U)]-glucosamine (0.36 Ci/mole) or 100 μCi of [5,6-^{3}H]-uridine (46 Ci/mole). Each group was incubated at 37°C for 24 hr at which time the incorporation was terminated by the addition of 2 ml of ice-cold RPMI 1640. Cell pellets were precipitated in 1% (w:v) phosphotungstic acid (PTA) in 0.5N HCl, washed twice in 10% (w:v) trichloroacetic acid (TCA), and extracted in 1 ml of 95% (v:v) ethanol and 1 ml of ethyl ether. The final acid-insoluble, lipid-extracted material was recovered by centrifugation and solubilized in 0.2 ml 1 N NaOH at 60°C. Protein content was assayed by the method of Lowry et al. (1951) and radioactivity was assessed by liquid scintillation counting. Data were calculated as mean values and standard deviations of quadruplicate samples in two or more experiments per determination and were expressed as a percentage of control group precursor incorporation. The kinetics of the recovery of cell growth and biosynthetic potential was determined in groups of L1210 cells which were exposed for 24 hr to increasing concentrations of tunicamycin, washed several times, and resuspended at 1.0×10^5 cells/ml in complete growth medium. At various intervals after the removal of the antibiotic, aliquots of control and treated cells were assessed in terms of growth rate and the incorporation of radiolabeled mannose or thymidine was measured as described previously.

Formation and determination of double-labeled, intracellular UDP-N-acetylglucosamine pools.

L1210 cells were incubated for 24 hr in the presence of 0.1, 1.0, and 10 μg/ml TM. Following treatment cells were washed thoroughly and 1×10^7 cells from each group were extracted with perchloric acid (PCA) (6% in 1 M ammonium formate, pH 4.4) for 10 min at 4°. The acid-soluble extracts were neutralized with 0.1 N KOH, centrifuged at 1200g, and aliquots of the supernatants were chromatographed by high-pressure chromatography (HPLC) as described previously (Bernacki et al., 1977). UDP-*D*-[6-^{3}H]-N-acetylglucosamine was used as an authentic standard for determining nucleotide retention time while the retention times of the other peaks were compared with those of authentic nucleotide standards. In order to confirm the accumulation of UDP-N-acetylglucosamine with TM treatment, L1210 cells were exposed to 1 or 3 μM TM and 0.75 μCi/ml of *D*-[^{14}C-(U)]-glucosamine (0.36 Ci/mmole). At intervals of 4, 12, and 24 hr later, nucleotide pool size analyses were again performed. Eluent fractions (0.5 ml) were collected from the column and analyzed for [^{3}H] and [^{14}C] by liquid scintillation counting. A comparison of labeled nucleotides and nucleotide sugar was made with untreated, labeled cells.

Lectin agglutination studies

L1210 cells (10^5 cells/ml) were incubated with various concentrations of TM (0.45 μg/ml, 1.5 μg/ml, and 4.5 μg/ml or approximately 0.5 μM, 1.8 μM, and 5.4 μM TM) in complete medium for 24 hr. Cells were harvested by centrifugation, washed twice with warmed (37°C) phosphate-buffered saline (PBS), pH 7.4, and resuspended at 2.0×10^6 cells/ml PBS. One milliliter of cell suspension was added to a prewarmed 2-ml cuvette. Wheat germ agglutinin (WGA) was added to a final concentration of 12 μg/ml. The cuvette chamber was kept at 37°C and optical density was monitored at 546 nm using a Beckman model 25 recording spectrophotometer. A 10–20 min lag period was noted before commencement of agglutination and agglutination was monitored for 40 min.

SDS-polyacrylamide gel electrophoresis

Plasma membrane fractions were prepared by the method of Hourani et al. (1973) from cells which were exposed to various concentrations of tunicamycin or *D*-glucosamine. Aliquots of each plasma membrane fraction were subjected to vertical polyacrylamide slab gel electrophoresis as described by Laemmli (1970) and modified by Guengerich (1978). The relative mobilities of the Coomasie-stained proteins were assessed by densitometric scanning of the gels with a Transidyne model 2955 scanning densitometer, and molecular weights were determined by comparison to the migration of protein standards (myosin, 200,000 D; β-galactosidase, 116,500 D; phosphorylase B, 94,000 D; bovine serum albumin, 68,000 D; and ovalbumin, 43,000 D).

Electron microscopy

L1210 cells exposed to tunicamycin (10 μM) or *D*-glucosamine (5 mM) for 24 or 48 hr were harvested, washed three times in phosphate-buffered saline at 4°, and the final pellet was resuspended in 3% phosphate buffer glu-

TABLE 1. The effects of tunicamycin on L1210 macromolecular precursor incorporation

Parameter	Incorporation[1] (% control ± S.D.) 1 μM TM	3 μM TM
[^{5}H]-leucine	96.6 ± 7.4	95.1 ± 6.9
[^{3}H]-thymidine	107.6 ± 1.7	99.3 ± 3.9
[^{3}H]-uridine	116.9 ± 15.5	160.6 ± 8.5
[^{3}H]-mannose	60.6 ± 5.4	22.5 ± 1.3
[^{3}H]-fucose	60.6 ± 4.7	24.8 ± 1.8
[^{14}C]-glucosamine	73.2 ± 6.3	47.5 ± 2.7

[1]L1210 cells were incubated in RPMI 1640 containing 10% serum, the indicated radiolabeled precursor and tunicamycin (TM) for 24 hr at 37°C. Incorporation was measured as described in Materials and Methods and the results were expressed as percentage control. All control values for DPM/mg protein exceeded 40,000.

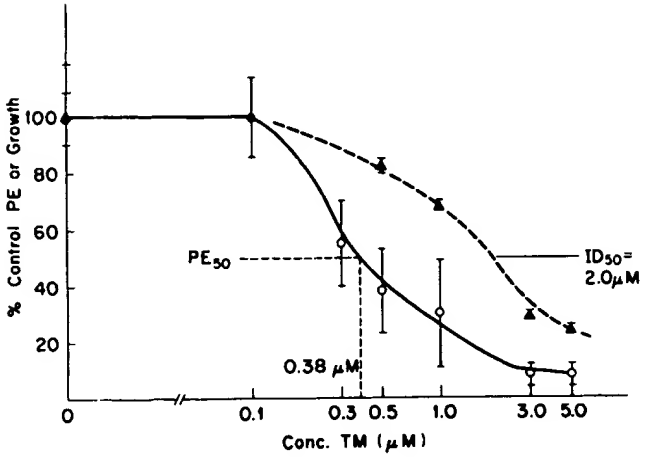


Fig. 1. Dose response for the inhibition of cell growth (–) and the inhibition of plating efficiency (PE) or colony formation in agarose (o–o) for L1210 leukemic cells exposed to TM for 48 hr in vitro. Colony formation in soft agar was assessed in the absence of TM following the initial 48-hr incubation of cells in liquid growth medium containing TM.

taraldehyde, pH 7.3, for 1 hr. The cell pellet was washed overnight in phosphate-buffered sucrose, postfixed in 1% (w:v) phosphate-buffered osmium tetroxide for 3 hr, dehydrated through a graded ethanol and propylene oxide series, and embedded in an Epon-araldite mixture. Thin sections (900 Å) were cut using a Porter-Blum MT-1 ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a Siemens Elmiskop 101.

Materials and reagents

Tunicamycin was obtained from Dr. Robert Hamill, Eli Lilly and Co. (Indianapolis, IN) and through Dr. John Douros of the National Cancer Institute. All cell culture materials were obtained through Grand Island Biological Co., (Buffalo, NY) and all materials for electron microscopy were purchased from Electron Microscopy Services (Fort Washington, PA). Recrystallized and deionized acrylamide and bis-acrylamide, as well as molecular weight protein standards, were obtained through Pharmacia Fine Chemicals (Piscataway, NJ). [Methyl-^{3}H]-thymidine and [5,6-^{3}H]-uridine were purchased from ICN Chemical and Radioisotope Division (Irvine, CA) and all other radioisotopes were obtained from New England Nuclear (Boston, MA). All other chemicals and reagents were of reagent grade or better and were obtained through Sigma Chemical Co. (St. Louis, MO).

RESULTS

Effects of tunicamycin on cell growth and macromolecular biosynthesis

The exposure of logarithmically growing L1210 cells to 1 μM tunicamycin for 24 hr resulted in a substantial reduction in the incorporation of *D*-mannose and *L*-fucose into glycoproteins without significant reduction in the incorporation of tritiated *L*-leucine or thymidine into protein or DNA (Table 1). The incorporation of *L*-fucose, which may represent the synthesis of complex-type asparagine-linked oligosaccharides, was inhibited to the same extent as that of *D*-mannose. The incorporation of *D*-glucosamine was not inhibited to as great an extent and this may have indicated that this monosaccharide was also incorporated into tunicamycin-insensitive *O*-linked oligosaccharides. At 3 μM tunicamycin, the differential inhibition of glycoprotein biosynthesis, in the absence of a significant reduction in protein synthesis, was essentially the same as that for exposure at the lower 1-μM concentration (Table 1).

Following longer periods of exposure (48 hr) over the same range of concentrations, a significant inhibition of both cell growth and survival was apparent. Tunicamycin inhibited L1210 cell growth in RPMI 1640 medium by 50% (ID_{50} value) at 2.0 μM (Fig. 1). Following this 48-hr exposure of L1210 cells to various concentrations of TM their ability to grow and form colonies in soft agar also was investigated. These studies indicated that treatment of cells with TM at 0.38 μM resulted in a 50% decrease in plating efficiency (PE_{50}) or soft agar colony formation. Examination of both dose-response curves revealed that neither was fully asymptotic with the x-axis, suggesting that 10–30% of the total cell population was not lethally affected during exposure to tunicamycin at concentrations as high as 5 μM.

The possibility of a heterogeneous response to the toxic effects of a the antibiotic led to a question as to whether the recovery of cells following the removal of tunicamycin was due to the recovery of the biosynthetic potential of the population as a whole or, alternatively, due to the rapid expansion of a subpopulation of cells which was refractory to tunicamycin. Studies were undertaken in which the recovery kinetics for both cell proliferation (thymidine incorporation and growth rate) and glycoprotein biosynthesis (mannose incorporation) were examined simultaneously in cell populations exposed to various concentrations of tunicamycin for 24 hr. It was demonstrated that recovery of the capacity to incorporate mannose and thymidine, like the recovery of cell growth itself, occurred almost immediately in cells which had been exposed to 1 μg/ml (1.2 μM) of the antibiotic (Fig. 2). At higher concentrations (3 μg/ml or 3.6 μM), however, it was apparent that the capacity of treated cells to incorporate mannose was nearly equal to that of control cells by 24 hr but that the inhibition of the incorporation of thymidine persisted. This differential effect was also apparent at the highest concentration of tunicamycin employed (9 μg/ml or 10.8 μM). These results suggested that the recovery in the capacity of the cell population to incorporate mannose was obligatory for the recovery of cell growth and that an expansion of a tunicamycin-insensitive subpopulation could not account

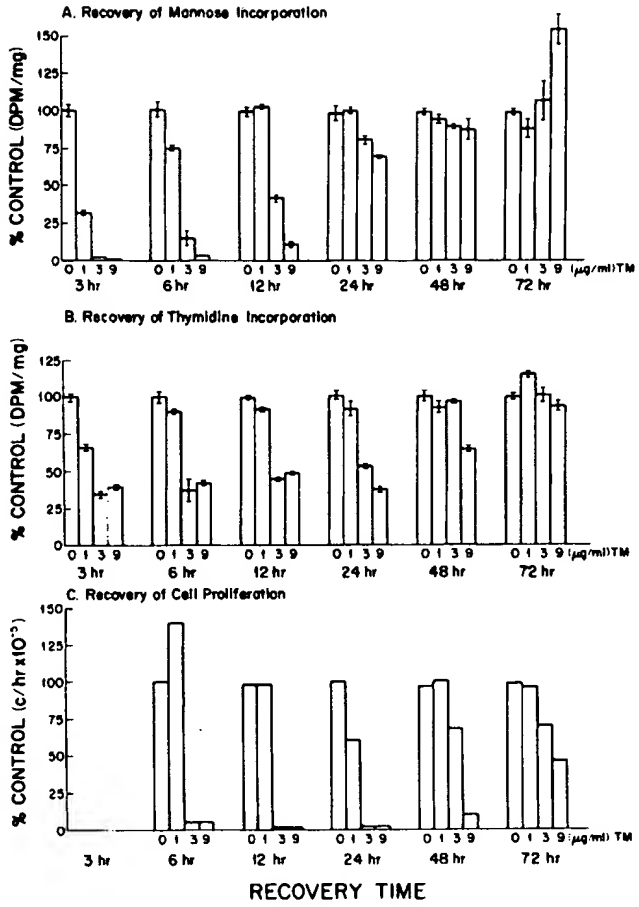


Fig. 2. Recovery of L1210 cells to incorporate [^{3}H]-mannose (A) and [^{3}H]-thymidine (B) and proliferate (C) following a 24-hr treatment with the given concentrations of TM in vitro.

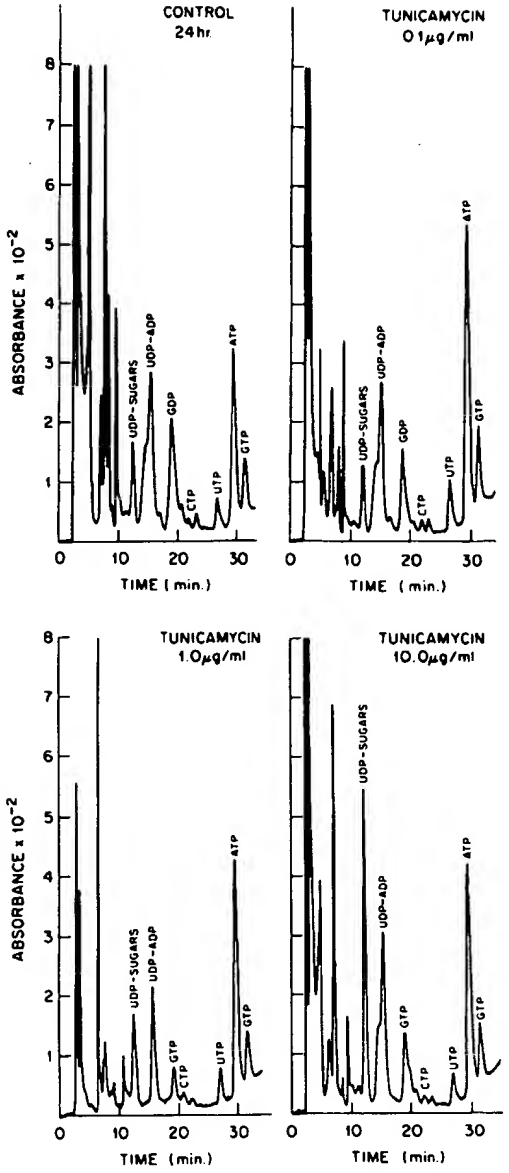


Fig. 3. High-performance liquid chromatography (HPLC) of intracellular nucleotide pool sizes of L1210 cells treated for 24 hr with 0.1, 1.0, and 10 μM/ml TM.

for the recovery of the biosynthesis of glycoproteins in the total cell population.

Accumulation of UDP-N-acetylglucosamine following the administration of tunicamycin

Based on the results of studies which demonstrated that radiolabeled uridine was incorporated to a greater extent in tunicamycin-treated cells (Table 1), the possibility that uridylates were being trapped intracellularly as nucleotide-sugar substrates was investigated. Initial analyses of the PCA-soluble metabolites of cells exposed to increasing concentrations of tunicamycin for 24 hr revealed dose-dependent increases in UDP-sugars with a threefold increase at 1.0 μM and over a fivefold increase at 10 μM (Fig. 3). Direct evidence that the observed changes in the nucleotide sugar pools was due to an accumulation of UDP-N-acetylglucosamine, the water-soluble substrate involved in the tunicamycin-sensitive formation of dolichol pyrophosphoryl N-acetyl-glucosamine, was obtained from studies in which cells were incubated concurrently with the antibiotic, [^{3}H]-uridine, and [^{14}C]-glucosamine and were then extracted with PCA at given intervals thereafter. The results of these studies revealed both dose-dependent and time-dependent accumulations of a double-labeled metabolite which cochromatographed with authentic UDP-[^{3}H]-N-acetylglucosamine. At the 4-hr time point, a number of [^{3}H]-uridine metabolites were present in control and treated cells and one major metabolite of [^{14}C]-glucosamine, which coeluted with UDP-[^{3}H]-N-acetylglucosamine in fractions 9–12, was also apparent (Fig. 4A). By 12 hr, most of the [^{3}H]-uridine metabolites had pulsed through the cells and a dose-dependent increase in the double-labeled metabolite with a higher specific activity in terms of [^{14}C]-glucosamine was apparent (Fig. 4B). By 24 hr, UDP-N-acetylglucosamine was the predominant labeled metabolite for all treatment groups but was present in the greatest amounts in cells exposed to the highest concentration (3 μM) of tunicamycin (Fig. 4C). These results suggested that the formation of UDP-N-acetylglucosamine was equiva-

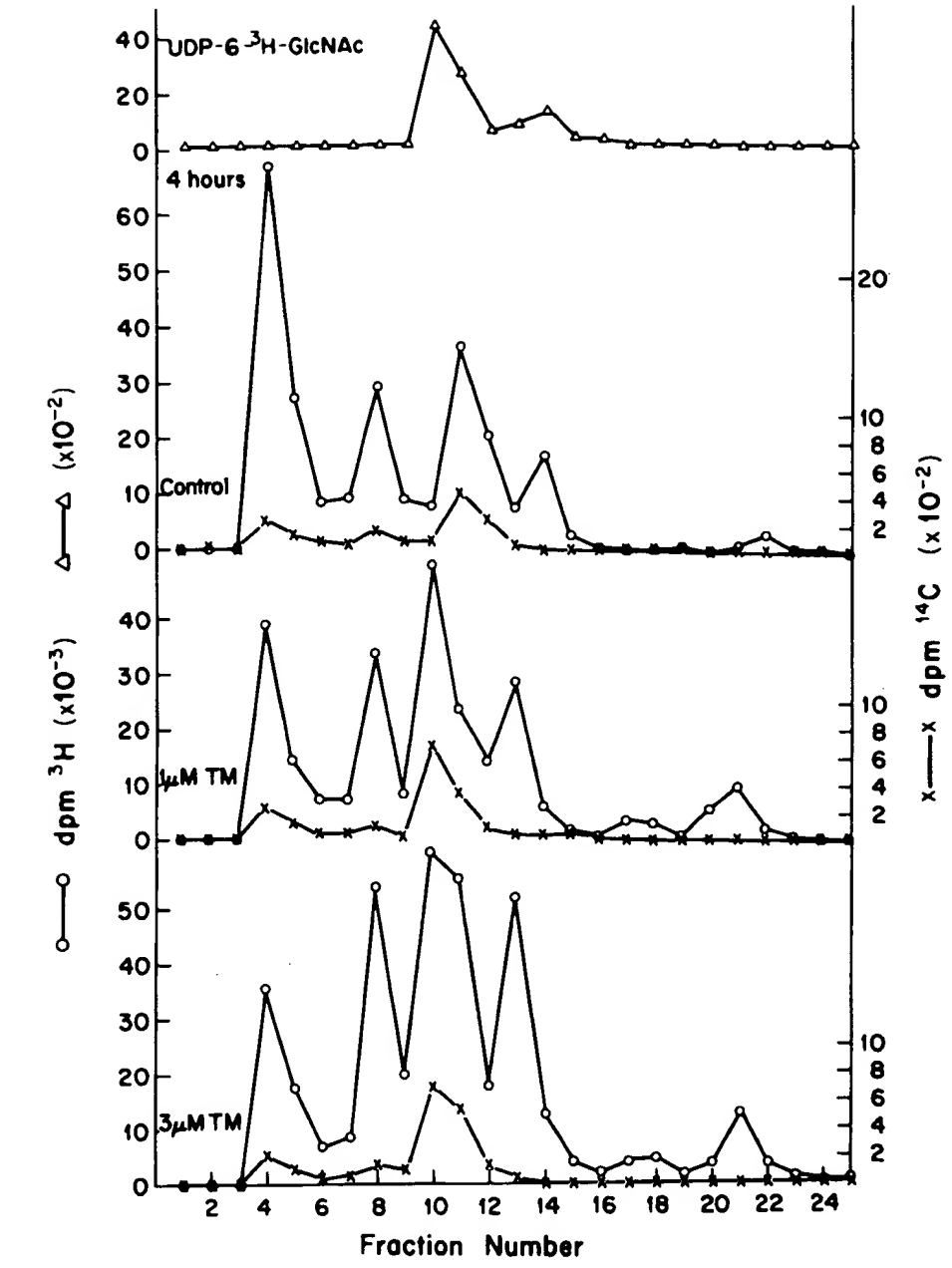
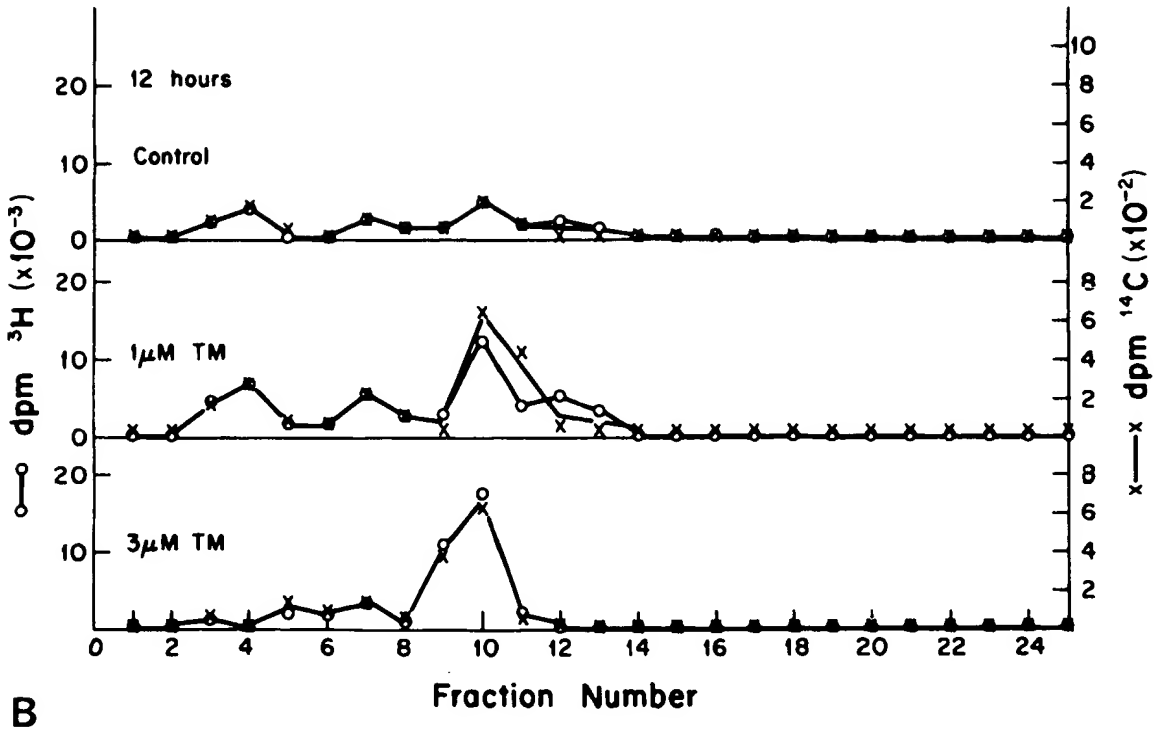


Fig. 4. High-performance liquid chromatography elution profiles of authentic UDP-[^{3}H]-N-acetylglucosamine and the acid-soluble intracellular metabolites from L1210 leukemic cells exposed to TM and incubated for 4 hr (A), 12 hr (B), and 24 hr (C) with [^{3}H]-uridine and [^{14}C]-glucosamine as described.


12 hours
Control
1μM TM
3μM TM
dpm ³H (x10⁻³)
x—x dpm ¹⁴C (x10⁻²)
Fraction Number
B

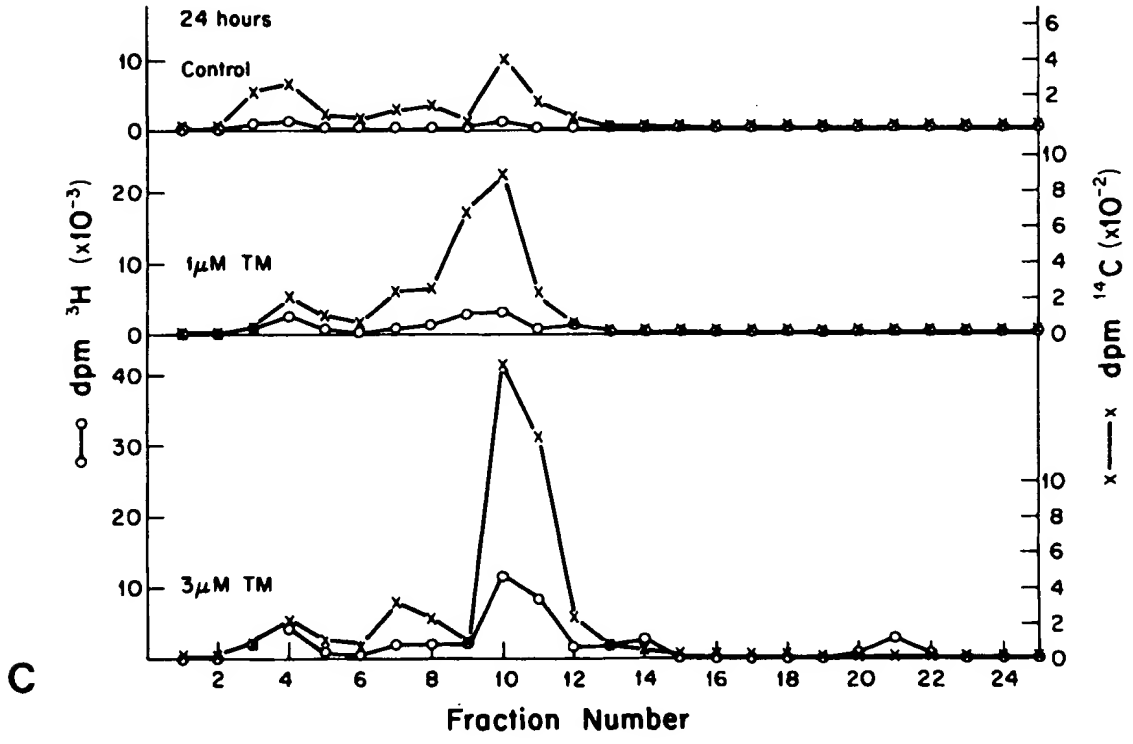

24 hours
Control
1μM TM
3μM TM
dpm ³H (x10⁻³)
x—x dpm ¹⁴C (x10⁻²)
Fraction Number
C

Figure 4.

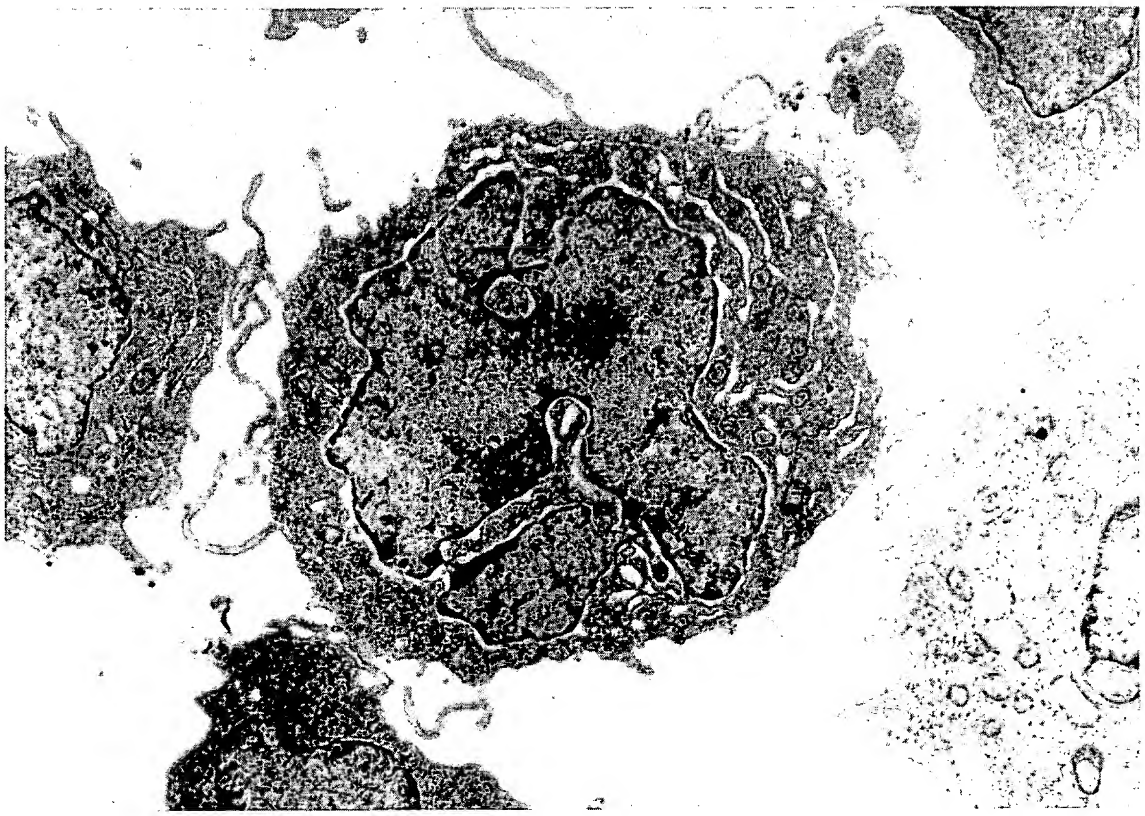


Fig. 5. An electron micrograph demonstrating the ultrastructural damage of L1210 leukemic cells exposed to 10 μM TM for 24 hr. Significant distentions of the endoplasmic reticulum and nuclear envelope are evident. × 9,000.

lent in both control and tunicamycin-treated cells but that it was not used as a sugar donor to a similar extent in the presence of the antibiotic.

Effect of tunicamycin and D-glucosamine on L1210 leukemia cell ultrastructure

The administration of high levels of aminosugars, such as glucosamine and galactosamine, causes elevations in the levels of intracellular nucleotide sugars and results in cytotoxicity (Decker and Keppler, 1974). Therefore a comparison of the effects of tunicamycin and *D*-glucosamine, administered at doses which produce similar elevations in intracellular nucleotide sugar pools, was performed with L1210 leukemia cells in vitro.

Treatment with 10 μM tunicamycin for 24 hr resulted in distentions of the endoplasmic reticulum and of the nuclear envelope (Fig. 5). When L1210 leukemia cells were exposed to equitoxic concentrations of *D*-glucosamine (5 mM) for 48 hr, the effects on cellular ultrastructure (Fig. 6) were remarkably similar to those induced by tunicamycin treatment; dilation of the endoplasmic reticulum and the nuclear envelope took place. Taken together, these studies suggested that the ultrastructural effects of exposure to toxic concentrations of either tunicamycin or *D*-glucosamine could be related to increases in the intracellular pools of UDP-N-acetylglucosamine.

Differentiation between the biochemical consequences of the exposure of L1210 cells to tunicamycin or *D*-glucosamine

Examination of the biochemical and cellular effects of *D*-glucosamine revealed several fundamental differences by which its activity could be distinguished from that of tunicamycin. Exposure to 1 or 3 mM *D*-glucosamine resulted in the partial inhibition of both protein and DNA synthesis whereas the incorporation of [^{3}H]-mannose into glycoproteins was not inhibited under the same conditions (Table 2). This result, which was essentially opposite to that obtained during the exposure of L1210 cells to tunicamycin, suggested that the accumulation of UDP-N-acetylglucosamine could not be the sole basis for the reversible inhibition of the synthesis of asparagine-linked oligosaccharides in the presence of the antibiotic. Further evidence for major differences in the biochemical effects of the two agents was obtained in studies in which the biochemical composition of the plasma membranes of tunicamycin or *D*-glucosamine-treated cells was examined following exposure to either agent at equitoxic concentrations for 48 hr. Evaluation of the densitometric scans of the Coomassie-stained SDS-polyacrylamide gel patterns revealed specific modifications resulting from tunicamycin treatment (Fig. 7A) which were not apparent in the plasma membranes derived from cells treated with *D*-glucosamine (Fig. 7B). Among the major alterations in membrane pro-

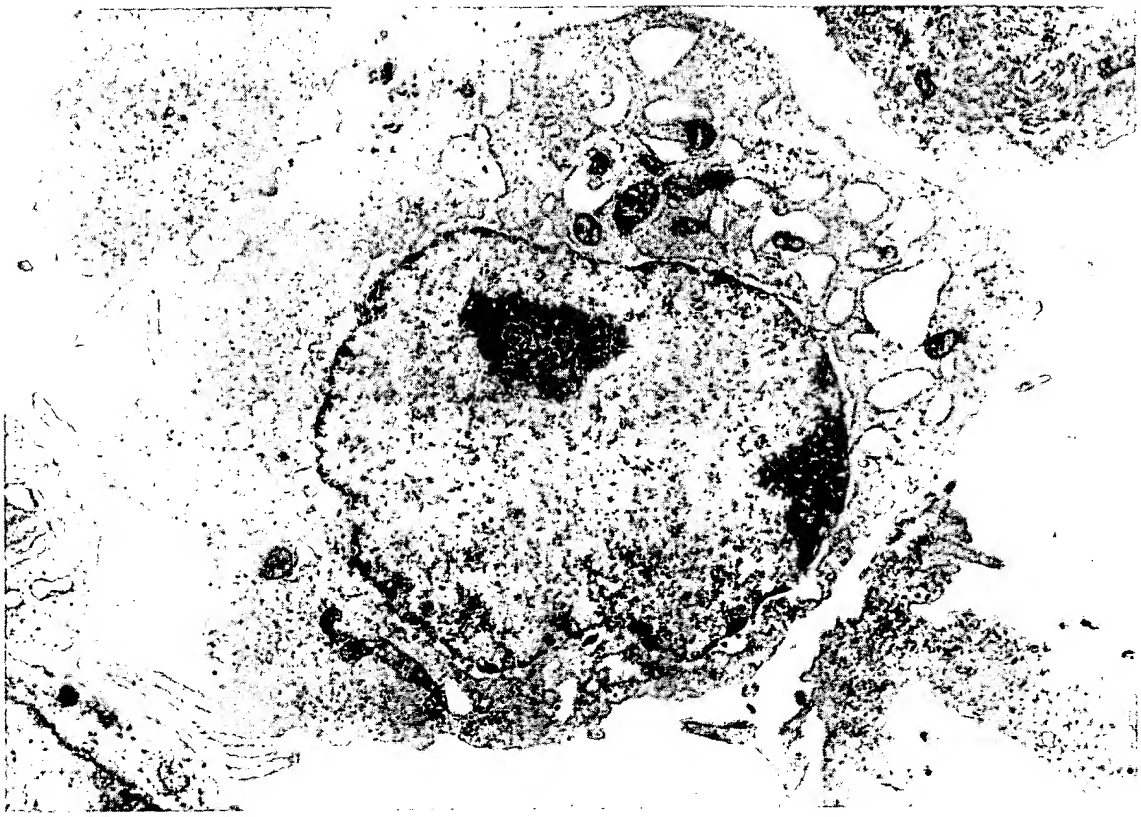


Fig. 6. Electron micrograph of a L1210 leukemia cell exposed to 5 mM *D*-glucosamine for 48 hr in vitro. Changes in endoplasmic reticulum and nuclear envelope are clearly evident and similar to those seen in Figure 5. × 9,660.

TABLE 2. Precursor incorporation into L1210 cell macromolecules following exposure to *D*-glucosamine

	% Control incorporation ± S.D.[1]	
Precursor	1 mM *D*-glucosamine	3 mM *D*-glucosamine
[^{3}H]-leucine	94 ± 5	82 ± 2
[^{3}H]-thymidine	93 ± 3	73 ± 3
[^{3}H]-mannose	97 ± 9	100 ± 10

[1]L1210 cells were incubated in RPMI 1640 containing 10% serum, the indicated radiolabeled precursor and *D*-glucosamine for 24 hr at 37°C. Incorporation was measured as described in Methods and the results were expressed as percentage control. All control values for DPM/mg protein exceeded 50,000.

teins after 1 μM tunicamycin were quantitative increases in protein or glycoprotein bands with apparent molecular weights of 44,000–45,000 and 53,000 and a decrease in the presence of a band with an apparent molecular weight of 47,000 (Fig. 7A). Exposure to 3 mM *D*-glucosamine for the same duration resulted in a single detectable modification involving an increased amount of a protein with an apparent molecular weight of 70,000 (Fig. 7B). A functional difference in the membrane-modifying activities of the two agents was further implied by differences found in the lectin agglutination assays (Fig. 8). L1210 leukemic cells treated with low amounts of wheat germ agglutinin (WGA) began to agglutinate after 10 min and were agglutinated within 25 min. Cells treated with low amounts of TM (0.45 μg/ml or 0.54 μM) for 24 hr demonstrated a lag in the commencement of agglutination (28 min) while cells treated at higher levels (1.5 and 4.5 μg/ml or 1.8 and 5.4 μM) became refractory to agglutination with WGA. Exposure of L1210 cells to *D*-glucosamine (5 mM) for 24 hr did not result in any changes in the WGA agglutination patterns of these cells (data not shown).

DISCUSSION

Exposure of murine L1210 cells to tunicamycin in vitro resulted in specific decreases in the incorporation of *D*-mannose, *L*-fucose, and *D*-glucosamine, altered cellular ultrastructure, and finally inhibition of cellular growth. Examination of the reversibility of the antiproliferative effect of the antibiotic revealed that recovery of cell growth was preceded by recovery of the biosynthesis of glycoproteins.

Following the exposure of L1210 cells to tunicamycin an increase in the intracellular pool of UDP-N-acetylglucosamine was observed. The role of the accumulation of this nucleotide sugar, which is a known feedback inhibitor of hexosamine production and, consequently, glycoprotein biosynthesis (Kornfeld et al., 1964), was examined in parallel experiments in which cells were exposed to *D*-glucosamine at levels which induced similar increases in UDP-N-acetylglucosamine pools (Bernacki et al., 1977). The treatment of cells with either tunicamycin or *D*-glucosamine resulted in similar, specific alterations in cellular ultrastructure, namely, distention of the endoplasmic reticulum and nuclear envelope. The effects of the two agents

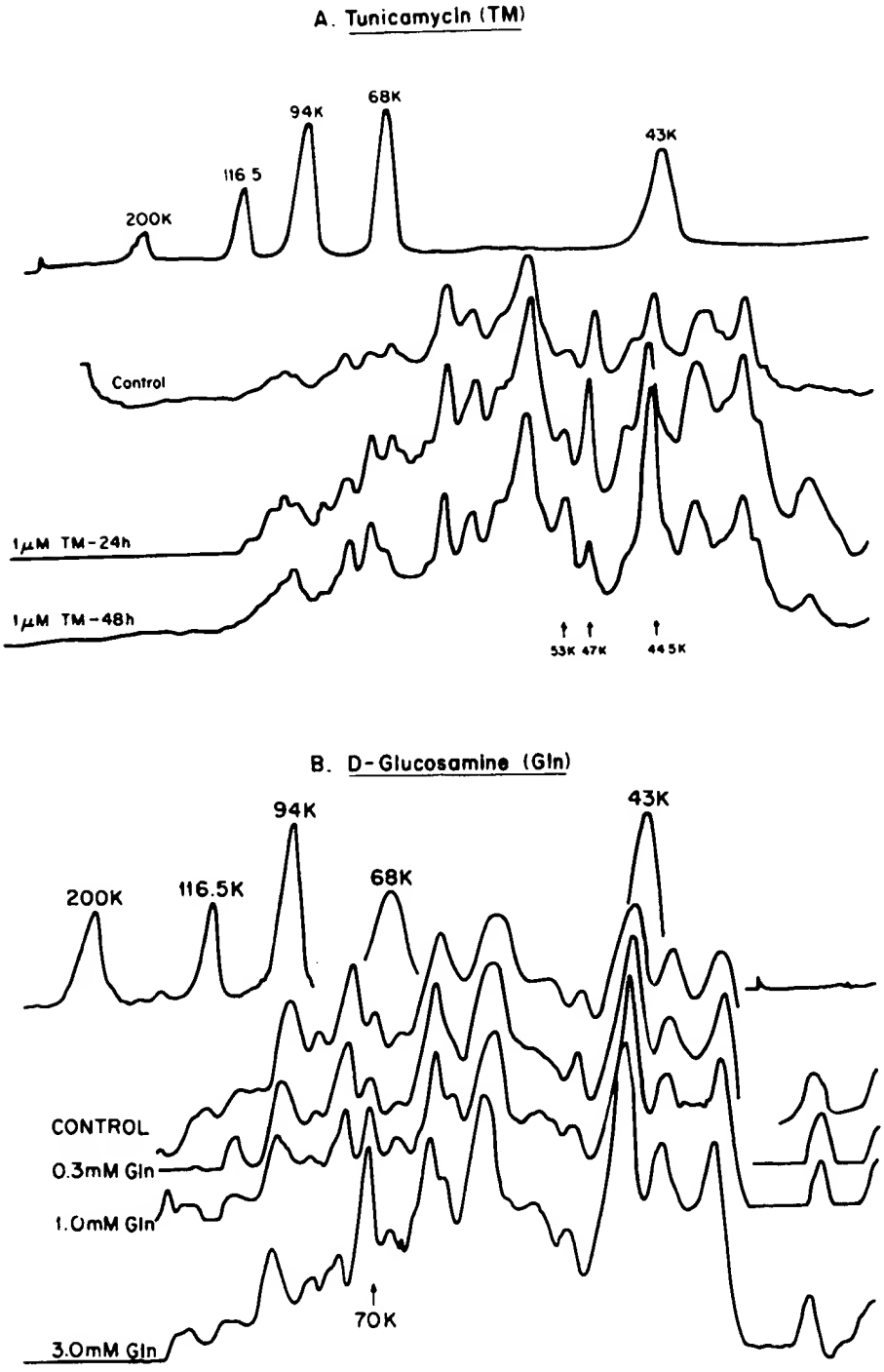


Fig. 7. A densitometric representation of the Coomassie blue staining patterns from SDS-polyacrylamide gel electrophoresis (PAGE) of plasma membrane fractions from L1210 leukemic cells exposed to (A) 0 or 1 μM for 24 or 48 hr and (B) 0, 0.3, 1.0, and 3.0 mM *D*-glucosamine for 48 hr in vitro.

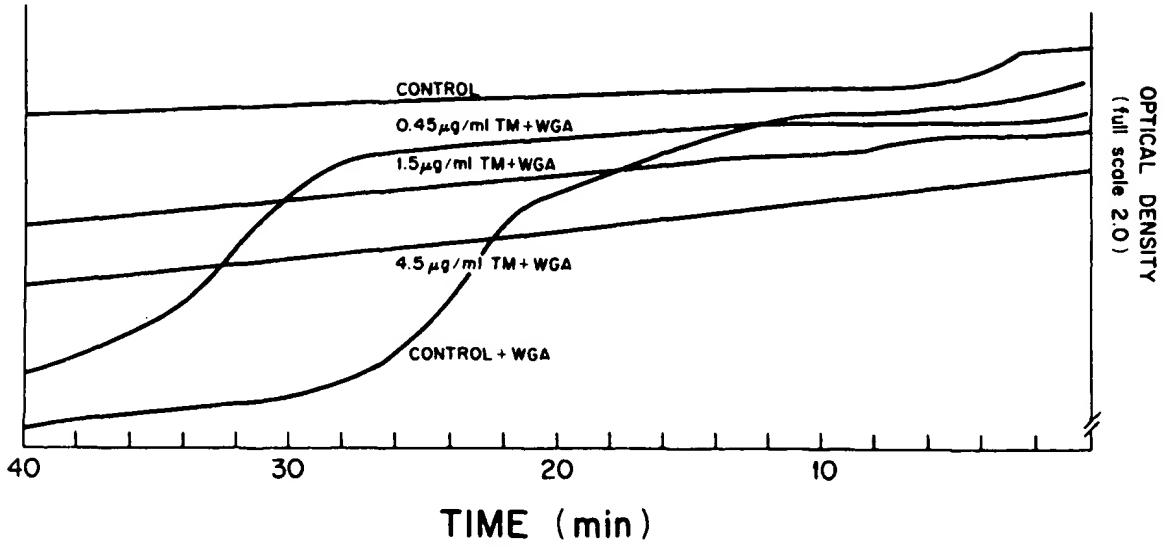


Fig. 8. Spectrophotometric analysis of wheat germ agglutinin (WGA)-induced agglutination of L1210 leukemia cells (2×10^6 cells/ml) treated with tunicamycin (TM) for 24 hr.

could, however, be distinguished by a number of other criteria, including patterns of inhibition of macromolecular biosynthesis, alterations in plasma membrane proteins, and cellular agglutination with wheat germ agglutinin. The results of these studies indicated that the direct inhibition of asparagine-linked glycoprotein biosynthesis by tunicamycin was the principal basis for the expression of TM cytotoxicity in L1210 leukemic cells and that the intracellular accumulation of UDP-N-acetylglucosamine, subsequent to exposure to the antibiotic, could account for some (swollen endoplasmic reticulum and nuclear envelope), but certainly not all, of its cytotoxicity.

Several earlier studies, including those of Quastel and Cantero (1953) and St. Arneault et al. (1971), had demonstrated that high concentrations of *D*-glucosamine could result in a loss of cell viability in several transplantable murine tumors. More recently, Friedman and Skehan (1980) demonstrated that in rat glioma cells exposure to high levels of *D*-glucosamine resulted in alterations in intracellular membrane structures and inhibition of lipid biosynthesis which led the authors to conclude that membrane was the target for the tumoricidal activity of this hexosamine. At present, the biochemical basis for membrane ultrastructural modifications by *D*-glucosamine or tunicamycin is not clear, but it is plausible that the intracellular accumulation of UDP-N-acetylglucosamine, as well as other nucleotide sugars, may result in the increased transport of these intermediates into the lumen of the endomembranes (Carey et al., 1980), thereby causing imbalances in vesicular osmolarity. Alternatively, the distention of the endoplasmic reticulum may have been the result of a direct inhibition of membrane components' biosynthesis by either tunicamycin or *D*-glucosamine, directly (Koch et al., 1979; Datema and Schwartz, 1979). Hercz and co-workers (1978) had demonstrated that hepatocytes from patients with an α_{-1} antitrypsin deficiency, which was related to the defective glycosylation of the asparagine-linked oligosaccharides of the protease inhibitor, gave evidence of an accumulation of the underglycosylated products in the distended lumen of the hepatocyte rough endoplasmic reticulum. Moreover, others have reported the accumulation of various secretory products and concurrent ultrastructural alterations following the treatment of cells with tunicamycin (Hickman et al., 1977).

We have demonstrated that *D*-glucosamine can induce tunicamycinlike alterations in cell ultrastructure without specific inhibition of glycoprotein biosynthesis. Thus, the possibility persists that in L1210 leukemic cells both the cumulative depletion of specific N-linked glycoproteins and secondary metabolic perturbations which occurred as a result of exposure to tunicamycin could each, to some extent, account for the cytotoxicity of this antibiotic. However, based on the tunicamycin recovery studies which suggested that the resumption of glycoprotein biosynthesis was a prerequisite for reinitiating cellular proliferation, we suggest that the direct inhibition of asparagine-linked glycoprotein biosynthesis by the antibiotic represents the major determinant in the expression of its cytotoxicity in vitro. *D*-glucosamine's cellular cytotoxicity, on the other hand, may be related more directly to uridylate trapping and the subsequent inhibition of a variety of metabolic pathways.

ACKNOWLEDGMENTS

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Inhibition of Experimental Pulmonary Metastasis of Mouse Colon Adenocarcinoma 26 Sublines by a Sialic Acid:Nucleoside Conjugate Having Sialyltransferase Inhibiting Activity

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ABSTRACT

The total and sialidase-releasable sialic acid contents of murine colon adenocarcinoma 26 sublines of high (NL-17) and low (NL-44) metastatic potential were found to be positively correlated with their ability to undergo metastasis. Furthermore, sialyltransferase activity of intact NL-17 cells was higher than that of NL-44 cells. These findings suggest that sialic acid on the cell surface may play a role in the metastasis of these cells.

Therefore, the effect of a sialyltransferase inhibitor, 5-fluoro-2′,3′-isopropylidene-5′-*O*-(4-*N*-acetyl-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-glycero-α-D-galactooctapyranosyl)uridine (KI-8110), on the experimental lung metastasis of NL-17 or NL-44 cells was examined. KI-8110 inhibited the transfer of sialic acid to its endogenous acceptor in NL-17 and NL-44 cells. NL-17 or NL-44 cells were injected into the tail veins of mice, and the metastasis-inhibiting activity of KI-8110 was evaluated on the basis of both the lung weight and the number of pulmonary surface nodules about 3 wk after the tumor cell injection and of the survival ratio of mice inoculated with the tumor cells. Pretreatment of tumor cells with KI-8110 together with i.v. injection of KI-8110 caused significant inhibition of pulmonary metastasis of both NL-17 and NL-44 cells. Inhibition of metastasis and prolongation of survival were also observed on i.v. injection of KI-8110 without pretreatment of the tumor cells with KI-8110, but the degree of inhibition was lower than that in the case of the two treatments together.

KI-8110 itself had neither cytostatic nor cytotoxic effects on NL-17 and NL-44 but reduced the retention of tumor cells in the lungs. This antimetastatic effect of KI-8110 may be due to modification of the tumor cell surface resulting from inhibition of sialyltransferase by KI-8110. In addition, a β-linked sialic acid:nucleoside conjugate (KI-8111) and an equimolar mixture of KI-8110 and KI-8111 (KI-414) also inhibited the metastatic ability of NL cells to the same extent as KI-8110 did.

INTRODUCTION

Cancer metastasis is one of the most important problems in cancer research. Metastatic processes are very complicated because they involve various factors, and the overall mechanism is not yet understood.

Recently biochemical studies have provided considerable evidence suggesting differences in the tumor cell surface properties between metastatic and nonmetastatic cells. Some of these findings indicate the close correlation of the sialic acid content of cell membranes with the metastatic potential. Bosmann *et al.* (1) and Yogeeswaran *et al.* (2) have found that the amount of neuraminidase-releasable sialic acid on the cell surface of a B16 melanoma subline of high metastatic potential was greater than that of a subline of low metastatic potential. Tao and Burger (3) have shown that a lectin-resistant B16 variant which had lost the experimental metastatic properties also showed a decrease in cell surface sialic acid content. Parallel findings have been observed for RNA tumor virus-transformed sarcoma lines (4) and other systems (5–9). Also reported was evidence which suggests a connection between the sialyltransferase activity of tumor cells and metastatic potential (10).

Thus sialylation of the tumor cell surface seems to be closely related to the metastatic potential of the tumor cells. Consequently it may be possible to modify the metastatic potential of tumor cells by altering the sialic acid metabolism of the cells. As reported in the previous paper, certain sialic acid:nucleoside conjugates show sialyltransferase inhibiting activity in normal murine lymphocytes (11, 12).

In this paper, we describe the effect of these sialic acid: nucleoside conjugates on tumor cell metastasis.

MATERIALS AND METHODS

Reagents. The sialic acid:nucleoside conjugate (KI-8110[2]) (Fig. 1), the β-anomer of KI-8110 (KI-8111), and an equimolar mixture of the α- and β-anomers of the above sialic acid:nucleoside conjugate (KI-414) were synthesized by the method of Kijima *et al.* (11). CMP:*N*-acetyl-[4,5,6,7,8,9-^{14}C]neuraminic acid (247 mCi/mmol) and [6-^{3}H]thymidine (15 Ci/mmol) were purchased from New England Nuclear (Boston, MA); neuraminidase (*Arthobacter ureafaciens*), from Nakarai Chemicals, Ltd. (Kyoto, Japan); trypsin (1:250), from DIFCO Laboratories (MI); and heparin sodium from Novo (Denmark).

Mice. Eight-wk-old female BALB/c mice were obtained from Charles River Japan (Kanagawa, Japan) and kept under pathogen-free conditions.

Tumor Cells. Tumor cell lines named NL-17 and NL-44 were established from colon adenocarcinoma 26 by Tsuruo *et al.* (13) and kindly provided by Dr. T. Yamori (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan). NL-17 is a subline that shows high pulmonary metastatic potential on i.v. injection, but NL-44 shows low metastatic potential under the same conditions. Cells were maintained *in vitro* in RPMI-1640:FCS. The generation time of NL-17 and NL-44 cells is almost the same (NL-17, 21.2 ± 0.2 h; NL-44, 22.6 ± 2.2

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[2] The abbreviations used are: KI-8110, 5-fluoro-2′,3′-isopropylidene-5′-*O*-(4-*N*-acetyl-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-glycero-α-D-galactooctapyranosyl)uridine; KI-8111, 5-fluoro-2′,3′-isopropylidene-5′-*O*-(4-*N*-acetyl-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-glycero-β-D-galactooctapyranosyl)uridine; KI-414, an equimolar mixture of KI-8110 and KI-8111; FCS, 10% fetal calf serum.

Exhibit E

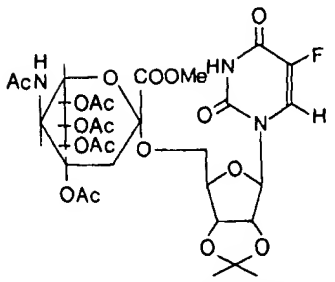

Fig. 1. Structure of KI-8110.

h), so that 24 h of pretreatment probably does not affect the two cell lines differently.

Assay of Sialyltransferase Activity. Tumor cells were harvested by trypsinization (0.05% trypsin:0.02% EDTA solution) for 2 min. The reaction was stopped by the addition of ice-cold RPMI-1640:FCS. The cells were washed twice with the medium and once with 0.01 M sodium phosphate buffer containing 0.12 M NaCl and 1 mM $MgCl_2$ (assay buffer). Cell numbers and cell viability were determined by the trypan blue exclusion test. Ectosialyltransferase activity was measured according to the method of Painter *et al.* (14). Briefly an incubation mixture containing 5×10^6 cells and 2 μM CMP:*N*-acetyl-[^{14}C]neuraminic acid in 100 μl of assay buffer was shown with or without the addition of 10^{-4} M KI-8110 at 37°C in a shaking water bath for 2 h, and the reaction was terminated by the addition of 2 ml of ice-cold 0.5 M HCl containing 1% phosphotungstic acid. The acid-insoluble materials were collected by centrifugation and then washed twice with 2 ml of phosphotungstic acid and once with 5% trichloroacetic acid. The washed pellet was extracted twice with chloroform:methanol (2:1, v/v) and once with chloroform:methanol (1:2, v/v). The extracts were pooled and dried in a scintillation vial, and the pellets were solubilized with Soluene-350 (Packard Instrument Company, Inc.). The radioactivity was determined with a liquid scintillation system.

Determination of Sialic Acid. Neuraminidase-releasable sialic acid was measured as follows. Tumor cells with or without pretreatment with 10^{-4} M KI-8110 for 24 h were washed twice with Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution and then detached from the culture dish by treatment with 0.02% EDTA for 5 min. The detached cells were then incubated with 0.02 units of neuraminidase in 1 ml of 0.01 M sodium phosphate buffer (pH 6.5) containing 0.15 M NaCl at 37°C for 2 h. After centrifugation, the supernatant was collected and assayed for sialic acid by the thiobarbituric acid method of Aminoff (15), and the pellet was assayed for protein by the method of Lowry *et al.* (16). Released sialic acid was not detectable in the absence of enzyme. Total sialic acid was determined as follows. Cells harvested as described above were suspended in 0.05 M H_2SO_4, and the suspension was kept for 1 h at 80°C. After centrifugation, the supernatant was assayed for sialic acid by the method of Roboz *et al.* (17), and the pellet was assayed for protein by the method of Lowry *et al.* (16).

Assay of Pulmonary Metastasis. NL-17 and NL-44 cells were cultured with (or without) 10^{-4} M KI-8110 for 24 h and harvested by trypsinization as described above. The final cell suspension contained 2.5×10^5 cells in 1 ml of 0.9% NaCl supplemented with 1% BALB/c mouse serum. The cell suspension (0.2 ml) was injected into the tail veins of mice, followed immediately by the injection of 0.2 ml of KI-8110 solution (2.5 mg/ml) or 0.9% NaCl solution. Then the mice were given 0.2 ml of KI-8110 solution (2.5 mg/ml) or 0.9% NaCl solution i.v. every 3 days. About 3 wk after the tumor cell implantation, the mice were subjected to autopsy, lung weights were determined, and, after fixation of the lungs in 10% formaldehyde:picric acid solution, pulmonary metastases were grossly estimated by counting the number of metastatic nodules on the pulmonary surface. It has been suggested that the "pulmonary i.v. colonization assay" does not truly reflect the state of spontaneous metastatic potential. For the sake of brevity, "pulmonary metastasis" in our experimental metastasis assay will mean i.v. implantation—survival and growth of NL cells.

Measurements of Cell Growth or Viability. Cells were incubated with or without KI-8110 in RPMI-1640:FCS at 37°C. At various culture times, the cell number and the cell viability were determined by trypan blue dye exclusion in a hemacytometer.

***In Vivo* Tumor Cell Retention.** Labeled tumor cells were injected into the tail veins of mice, and the retention of radioactivity in the lungs was studied. NL-17 cells were incubated with [^{3}H]thymidine for 24 h at a concentration of 25 μCi/ml. The labeled tumor cells were injected into the tail veins, and then at various times after the injection, mice were sacrificed and their lungs were excised. The excised lungs were put into scintillation vials, solubilized with NCS (Amersham, Arlington, IL), and then assayed for radioactivity with a liquid scintillation system.

RESULTS

Effects of KI-8110 on Sialyltransferase Activity in NL-17 and NL-44 Cells. When tumor cells were incubated with *N*-acetyl-[^{14}C]neuraminic acid in the presence of KI-8110, the incorporation of *N*-acetyl-[^{14}C]neuraminic acid into glycoproteins and glycolipids of the tumor cells was significantly inhibited (Table 1). In this assay, cell viability did not decrease during the incubation. Table 1 also shows that the incorporation of *N*-acetyl-[^{14}C]neuraminic acid was about 2-fold higher in NL-17 cells than in NL-44 cells. Since NL-17 is experimentally a highly metastatic cell line and NL-44 a cell line of low metastatic potential, sialyltransferase activity may be positively correlated to the metastatic potential of tumor cells.

Change in Sialic Acid Content of NL-17 and NL-44 Cells on Treatment with KI-8110. As shown in Table 2, the neuraminidase-releasable sialic acid and total sialic acid contents of the tumor cells decreased on treatment with 10^{-4} M KI-8110 for 24 h. For both NL-17 and NL-44, the effect of the drug on the neuraminidase-releasable sialic acid content of the cells was found to be more significant than that on the total sialic acid content. It is also shown in Table 2 that both the neuraminidase-releasable and total sialic acid contents are about 2-fold higher in NL-17 cells than in NL-44 cells. These results suggest that the sialic acid content is positively correlated with the metastatic potential in these cell lines like the sialyltransferase activity discussed above.

Effects of KI-8110 on Pulmonary Metastasis Resulting from i.v. Injection of NL-17 and NL-44 Cells. As shown in Table 3 and Fig. 2, pulmonary metastasis of NL-17 and NL-44 cells was significantly inhibited by pretreatment and i.v. injection of KI-8110. Increases in lung weight due to the growth of metastasized colonies were also inhibited by KI-8110. Administration of KI-8110 i.v. without pretreatment inhibited pulmonary metastasis, and the effect of the combination of pretreatment with KI-8110 with i.v. administration of the drug was additive, as shown in Table 3. The survival of mice inoculated with NL-17 cells was prolonged significantly by the administration of KI-8110 (Fig. 3). KI-8111, β-anomer of KI-8110, and KI-414, an equimolar mixture of KI-8110 and KI-8111, also inhibited the metastatic ability of NL cells to the same extent as KI-8110 did (Table 4). Furthermore, KI-8111 inhibited sialic acid transfer to the endogenous acceptors of NL-17 cells (Table 1). This suggests that the β-linked sialic acid:nucleoside conjugate as well as the α-linked sialic acid:nucleoside conjugate (KI-8110) has an inhibitory activity toward the metastasis of NL cells. KI-8110 (0.1 mM) had no effect on the growth and viability of NL-17 and NL-44 cells until 5 days after addition of KI-8110 (data not shown).

Table 1

Effect of KI-8110 and KI-8111 on sialic acid transfer to the endogenous acceptors of NL-17 and NL-44 cells

Cell line	Treatment	N-acetyl-[^{14}C]neuraminic acid incorporated (dpm/10^7 cells)			
		Protein		Lipid	
NL-17	None	3005.3 ± 116.5[a]	3157.6 ± 202.2	1047.3 ± 246.4	1275.7 ± 97.4
	KI-8110 (10^{-4} M)	2007.4 ± 473.2[b]		629.1 ± 34.5[b]	
	KI-8111 (10^{-4} M)		2689.2 ± 67.2[b]		978.5 ± 28.3[c]
NL-44	None	1398.9 ± 8.5	1816.3 ± 0.4	448.3 ± 24.0	693.9 ± 103.4
	KI-8110 (10^{-4} M)	892.7 ± 142.9[c]		272.4 ± 14.8[d]	
	KI-8111 (10^{-4} M)		1457.8 ± 32.7[d]		587.2 ± 13.8

[a] Mean ± SD for three experiments.
[b] Treatment group *versus* nontreated group, Student's *t* test, $P < 0.05$.
[c] Treatment group *versus* nontreated group, Student's *t* test, $P < 0.01$.
[d] Treatment group *versus* nontreated group, Student's *t* test, $P < 0.001$.

Table 2

Cell surface (neuraminidase-releasable) and total sialic acid contents of KI-8110-treated or nontreated NL-17 and NL-44 cells

Cell line	Treatment	Sialic acid (μg/mg protein)	
		Neuraminidase releasable	Total
NL-17	None	3.33 ± 0.22[a]	13.58 ± 1.69
	KI-8110 (10^{-4} M)	2.75 ± 0.15[b]	9.90 ± 1.72[c]
NL-44	None	1.53 ± 0.04	7.78 ± 1.17
	KI-8110 (10^{-4} M)	1.22 ± 0.04[d]	6.50 ± 0.25[c]

[a] Mean ± SD for three experiments.
[b] Nontreated group *versus* treated group, Student's *t* test, $P < 0.05$.
[c] Nontreated group *versus* treated group, Student's *t* test, $P < 0.1$.
[d] Nontreated group *versus* treated group, Student's *t* test, $P < 0.001$.

Table 3

Effect of KI-8110 on the experimental metastasis of NL-17 and NL-44 cells in mice

Cell line	Treatment	Lung wt (mg)	Pulmonary metastasis			
			No. of colonies			
			Mean ± SD	Median	Range	Incidence[a]
None	None	140 ± 26[b]	0	0	0	0/8
NL-17	None	520 ± 183	81 ± 26[c]	72	47–130	8/8
NL-17	KI-8110[d]	201 ± 103[e]	23 ± 19[e]	21	0–53	7/8
NL-17[f]	KI-8110[d]	165 ± 53[e]	8 ± 12[e]	2	0–34	5/8
NL-44	None	201 ± 47	37 ± 7[c, g]	35	29–52	8/8
NL-44	KI-8110[d]	161 ± 54	18 ± 12[h]	18	1–34	8/8
NL-44[f]	KI-8110[d]	138 ± 31[h]	10 ± 5[e]	10	2–17	8/8

[a] Number of mice showing metastasis per number of mice used.
[b] Mean ± SD.
[c] Nontreated NL-17 group *versus* nontreated NL-44 group, Student's *t* test, $P < 0.001$.
[d] Mice were given KI-8110 (0.5 mg/body) i.v. every 3 days after tumor cell inoculation.
[e] Nontreated group *versus* treated group, Student's *t* test, $P < 0.001$.
[f] Cells were pretreated with 10^{-4} M KI-8110 for 24 h before inoculation.
[g] The size of colonies was much smaller than that in the case of NL-17.
[h] Nontreated group *versus* treated group, Student's *t* test, $P < 0.01$.

Effect of KI-8110 on *In Vivo* Tumor Cell Retention. Thirty min after tumor cell injection, approximately 80% of nontreated NL-17 cells were retained in the lungs, but less than 50% of KI-8110-treated NL-17 cells injected with KI-8110 were retained in the lungs at this time. After 30 min, the number of cells retained in the lungs decreased with time. The retention time in the lungs was shorter for KI-8110-treated NL-17 cells than for nontreated cells (Fig. 4).

DISCUSSION

Metastasis occurs via a complex cascade of events or a series of sequential steps whereby tumor cells invade neighboring tissue and penetrate into the lymphatic and/or circulatory systems, become detached from the primary tumor mass, and spread to near and distant sites where they become arrested, invade, and finally proliferate to form new metastatic colonies (18). In this cascade, tumor cells interact with themselves; with a number of other cells, vascular, endothelial, and circulating host cells; and with soluble blood components. Such interactions may affect the metastatic nature of the tumor cells. Therefore, the tumor cell surface is one of the most important targets for research on tumor metastasis. In fact, Hagmar and Norrby (19) and Fidler (20) used trypsin to alter tumor cell surface properties and found that the metastasis potential was reduced by this treatment. Irimura *et al.* (21) used a cell glycosylation inhibitor, tunicamycin, to modify the biosynthesis of surface glycoproteins of B16 melanoma cells, and they found that the tunicamycin-modified B16 cells failed to form experimental pulmonary tumor colonies after i.v. injection.

Sialic acids are terminal sugars of cell surface glycoconjugates. Recently it has been suggested that the sialic acid content or sialyltransferase activity of the tumor cell surface is positively related to the metastatic potential of the tumor cells (1–10). However, some authors have failed to observe this correlation (22, 23). This inconsistency may be due to variations in cell lines. In the present study, it was observed that NL-17, a highly metastatic cell line when implanted i.v., did in fact show higher sialyltransferase activity and a higher sialic acid content than the cell line of low metastatic potential, NL-44. As we reported previously (12), the sialic acid:nucleoside conjugate, KI-8110, has a sialyltransferase inhibiting activity that specifically depends on the acceptor. Sialic acid transfer to O-glycosidically linked sugar chains of glycoproteins was specifically inhibited, and this inhibition was found to be long lasting when compared with the case of CDP, a known sialyltransferase inhibitor. KI-8110 inhibited the sialyltransferase activity of both NL-17 and NL-44 cells and decreased their sialic acid contents.

When mice were given i.v. injections of NL-17 or NL-44 cells pretreated with KI-8110 and then given KI-8110 i.v., pulmonary metastasis was significantly suppressed by these treatments in both NL-17 and NL-44 cells, and the survival of the mice was prolonged. Administration of KI-8110 alone i.v. also inhibited pulmonary metastasis, but the degree of inhibition was lower than that with the above combined treatment. Actually, the effects of the two treatments are additive.

KI-8110 itself had neither cytostatic nor cytotoxic effects on NL-17 and NL-44 cells, yet it reduced the retention of tumor cells in the lungs. Therefore, KI-8110 may have an effect at least on

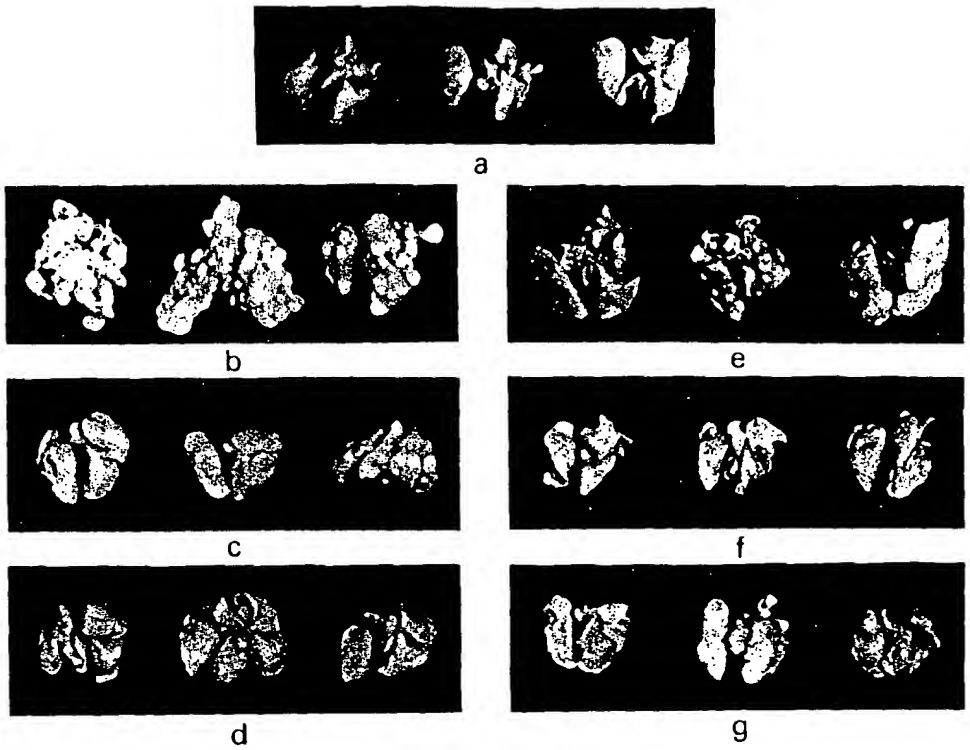

Fig. 2. Gross appearance of lungs from tumor cell-inoculated mice. In *a* are control mice; in *b*, mice were inoculated with NL-17 cells at 5×10^4 cells/mouse; in *c*, mice were inoculated with NL-17 cells at 5×10^4 cells/mouse and treated with KI-8110 at 0.5 mg/mouse/3 days; in *d*, mice were inoculated with NL-17 cells, which had been pretreated with 10^{-4} M KI-8110 for 24 h at 5×10^4 cells/mouse, and treated with KI-8110 at 0.5 mg/mouse/3 days; in *e*, mice were inoculated with NL-44 cells at 5×10^4 cells/mouse; in *f*, mice were inoculated with NL-44 cells at 5×10^4 cells/mouse and treated with KI-8110 at 0.5 mg/mouse/3 days; in *g*, mice were inoculated with NL-44 cells, which had been pretreated with 10^{-4} M KI-8110 for 24 h at 5×10^4 cells/mouse, and treated with KI-8110 at 0.5 mg/mouse/3 days. The lungs were excised from the mice 21 days after the i.v. injection of tumor cells and then washed and fixed with Bouin's solution.

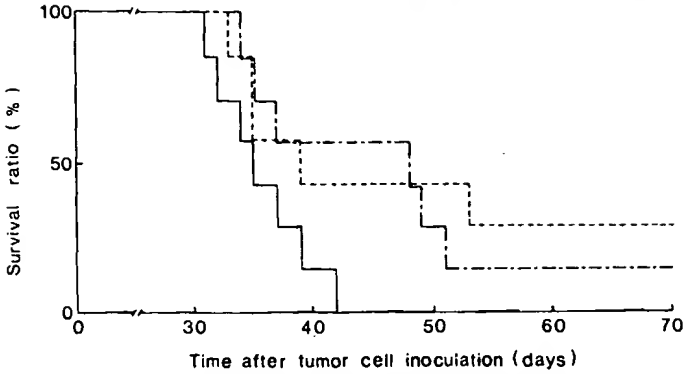

Fig. 3. Effect of KI-8110 on the survival ratio of mice inoculated i.v. with NL-17 cells. Mice were inoculated with NL-17 cells at 5×10^4 cells/mouse (——); NL-17 cells which had been pretreated with 10^{-4} M KI-8110 for 24 h, at 5×10^4 cells/mouse, and KI-8110 at 0.5 mg/mouse/3 days (– – – –); or NL-17 cells at 5×10^4 cells/mouse and KI-8110 at 1.0 mg/mouse/day (—— – ——), into the tail vein.

Table 4

Effect of KI-8110 and KI-8111 on the experimental metastasis of NL-17 cells in mice

Cell line	Treatment	Lung wt (mg)	Pulmonary metastasis: No. of colonies: Mean ± SD	Median	Range	Incidence[a]
None	None	127 ± 15[b]	0	0	0	5/5
NL-17	None	508 ± 285	53 ± 30	47	20–93	5/5
NL-17	KI-8110[c]	165 ± 74[d]	11 ± 11[d]	9	0–30	4/5
NL-17[e]	KI-8110[c]	130 ± 6[d]	1 ± 1[f]	0	0–2	2/5
NL-17	KI-8111[g]	221 ± 103	25 ± 20	23	8–57	5/5
NL-17[h]	KI-8111[g]	125 ± 10[d]	2 ± 3[f]	1	1–7	5/5

[a] Number of mice showing metastasis per number of mice used.
[b] Mean ± SD.
[c] Mice were given KI-8110 (0.5 mg/body) i.v. every 3 days after tumor cell inoculation.
[d] Nontreated group *versus* treated group, Student's *t* test, $P < 0.05$.
[e] Cells were pretreated with 10^{-4} M KI-8110 for 24 h before inoculation.
[f] Nontreated group *versus* treated group, Student's *t* test, $P < 0.01$.
[g] Mice were given KI-8111 (0.5 mg/body) i.v. every 3 days after tumor cell inoculation.
[h] Cells were pretreated with 10^{-4} M KI-8111 for 24 h before inoculation.

the initial events of the metastatic cascade, *i.e.*, tumor arrest. Since KI-8110 inhibits sialyltransferase, it is possible that this antimetastatic effect is due to modification of tumor cell surface properties affecting the sialic acid content of the cells. We are now studying the effect of KI-8110 on other metastatic cells, Lewis lung carcinoma, and B16 melanoma cells. Sinha and Goldbery (24) noted that neuraminidase treatment tends to change the distribution of metastatic colonization. Furthermore it was reported that the natural killer susceptibility of tumor cells decreased as the tumor cell surface sialic acid content increased (25), and it has been proposed that sialic acid is closely related to cell surface antigenicity and sometimes has a masking effect on tumor antigens (26, 27). These findings suggest that the change in sialic acid content on the tumor cell surface may affect the susceptibility of tumor cells to the host immune system.

Furthermore some metastatic tumor cells are known to contain a platelet-aggregating material, which is a trypsin-sensitive glycoprotein and sensitive to neuraminidase treatment (28, 29). Platelet-aggregating material activity decreased on treatment of NL-17 cells with KI-8110[3], and this might have resulted from the inhibition of tumor cell sialyltransferase by KI-8110. Since this tumor cell-induced platelet aggregation plays a crucial role in tumor cell arrest (30, 31), loss of platelet-aggregating material activity may decrease the metastatic potential of tumor cells.

Thus sialic acid can be said to be a key substance in tumor metastasis, and the antimetastatic effect of KI-8110 observed in the present study may possibly be related to the change in sialic acid metabolism on the tumor cell surface. It is possible that

[3] I. Kijima-Suda, Y. Miyamoto, S. Toyoshima, M. Itoh, and T. Osawa, unpublished data.

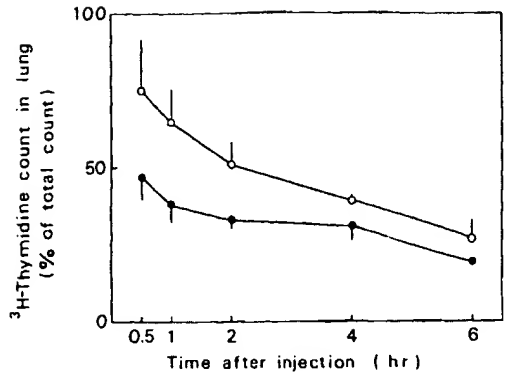

Fig. 4. Effect of KI-8110 on retention of NL-17 cells in the lung. Mice were given injections of either [^{3}H]thymidine-labeled NL-17 cells at 5×10^4 cells/mouse (○) or [^{3}H]thymidine-labeled NL-17 cells which had been pretreated with 10^{-4} M KI-8110 for 24 h, at 5×10^4 cells/mouse, and KI-8110 at 0.5 mg/mouse (●), into the tail vein. Mice were subjected to autopsy at the indicated times, the lung were removed, and the radioactivity in them was determined. *Points*, mean of two measurements; *bars*, SE.

other mechanisms may be responsible for the observed antimetastatic effect of KI-8110, *e.g.*, platelet aggregation, platelet-derived growth factor-induced growth of NL cells, or adhesion to fibronectin or collagen. Preliminary results suggest that KI-8110 decreases platelet aggregation and blocks platelet-derived growth factor-induced growth. These phenomena are possibly related to the sialic acid content on the cell surface. However, it has no effect on adhesion and does not induce cytotoxic T-lymphocytes or natural killer cells. Details will be published elsewhere.

We conclude that the sialic acid:nucleoside conjugate (KI-8110) has a potent antimetastatic effect on experimental metastasis, and that this effect may be due to a modification of tumor cell surface properties. The specific modification site(s) on the cell surface and the nature of the modification are currently under study.

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